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Luteinizing and Human Chorionic Gonadotropin Hormones Increase Intercellular Communication and Gap Junctions in Cultured Mouse Leydig Cells

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The effect of luteinizing (LH) and human chorionic gonadotropin (hCG) hormones on gap junctions (GJs) and intercellular communication (ic) was evaluated in Leydig (interstitial) cells from mouse testes. Cell cultures enriched in Leydig cells were studied under control conditions and when maintained in the presence of 100 ng/mL LH, 10 ng/mL hCG, or 1 m*M* dibutiryl-cAMP (db-cAMP), for 8, 24, and 36 h. To monitor the extent of ic, Lucifer yellow (LY) was injected through a patch pipet into one cell of-small cell aggregates (6–10), and its transfer was evaluated using fluorescent microscopy. The expression of GJs was monitored using immunofluorescent (IF) labeling of connexin 43 (Cx43) with a specific antibody. Testosterone secretion was determined by radioimmunoassay. At all culture times, testosterone levels in the medium were higher in treated than in control cell cultures. In cell cultures of 8 h, LY transferred to most of the neighboring cells (93%) and cell membrane appositions showed abundant Cx43; no difference was found between control and treated cells. In contrast, in control cell cultures of 24 and 36 h, LY transferred to a reduced fraction of neighboring cells (46 and 21%, respectively) and Cx43 labeling was markedly decreased. Addition of LH, hCG, or db-cAMP, to cell cultures for 24 and 36 h completely prevented the decrease in ic and Cx43 expression. Immunoblot studies, from total protein homogenates of cell cultures of 36 h, showed that relative levels of 40- and 43-kDa bands, characteristic of Cx43, were higher in treated than in control cells. These results demonstrate that the expression of Cx43 and ic in Leydig cells is modulated by LH and hCG, and suggest that their effect is mediated by the second messenger of these hormones, cAMP.

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Introduction

Intercellular communication (ic) through gap junctions (GJs) occurs between Leydig cells from the testis (Kawa, 1987). In recent years, five different GJ proteins, now termed connexins, Cx26, Cx32 (Zhang and Nicholson, 1989), Cx33, Cx37 (Haefliger et al., 1992), and Cx43 (Kadle et al., 1991; Risley et al., 1992; Pérez-Armendariz et al., 1994; Varanda and Campos de Carvalho, 1994), have been identified in various types of testicular cells from rat and mouse testis, using specific antibodies and/or cDNAs. However, only Cx43 has been localized in GJs formed between Leydig cells either *in situ* (Risley et al., 1992; Pérez-Armendariz et al., 1994) or in vitro (Pérez-Armendariz et al., 1994; Varanda and Campos de Carvalho, 1994). More recently, we (Pérez-Armendariz et al., 1994) and others (Varanda and Campos de Carvalho, 1994) found that macroscopic and unitary electrical properties of GJ channels between dissociated pairs of Leydig cells were closely similar to the ones found between cell pairs from other tissues, that naturally or in vitro-induced express mainly Cx43. Thus, we proposed that Cx43 was the main GJ protein expressed between Leydig cells. Here, we provide further evidence to support this contention by showing that the expression of Cx43 is required for the occurrence of direct cell-to-cell communication between Leydig cells.

GJ channels may carry signals that regulate hormonal secretion. Studies in secretory cells from other glands have shown that transient stimulation with secretagogues induces changes in biophysical properties of GJ channels (Itwatsuki and Petersen, 1977; Eddlestone et al., 1984; Neyton and Trautmann, 1986; Munari-Silem et al., 1991; Kanno et al., 1993) and that sustained stimulation with secretagogs induced an increase in the expression of GJs (Meda et al., 1979; In't Veld et al., 1985; Saéz et al., 1991;

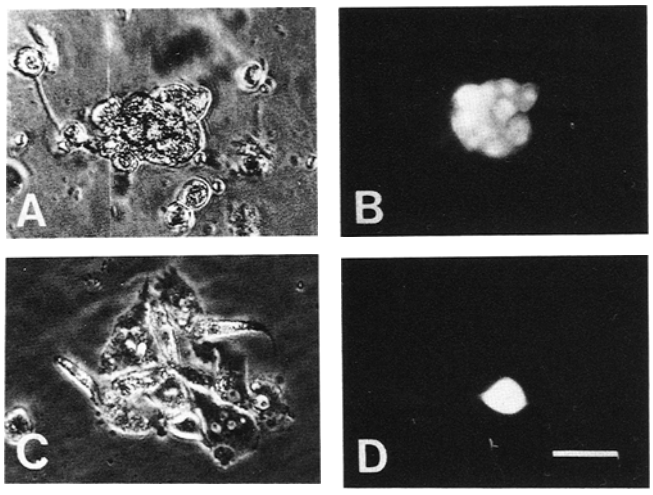

Fig. 1. Intercellular communication in Leydig cells decreases with culture time. **(A, C)** Phase-contrast and **(B, D)** fluorescent micrographs of representative Leydig cells cultured for 8 (A, B) and 36 h (C, D). Leydig cells freshly dissociated were rounded (A) and become elongated after being maintained in culture for 36 h (C). At both culture times, cells were identified by their abundant lipid droplets that were visualized using phase-contrast microscopy as birefringent bodies (arrows) (A, C). LY (4% LiCl 1 m*M*, pH 7.16) was injected by diffusion through a patch pipet for 5 min into one cell of cell clumps (6–10 cells), mainly formed by Leydig cells. After this time, in cell clumps cultured for 8 h, LY transferred to most of its neighbors (B), whereas in cell clumps cultured for 36 h, no dye transfer was detected (D). Calibration bar 20 μm.

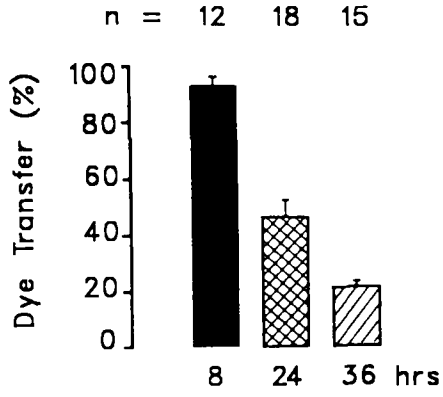

Fig. 2. Percentage of decay in dye coupling between Leydig cells with time in culture. Bar amplitude represents the mean percent of dye-coupled cells detected in groups of cell clumps cultured for 8, 24, and 36 h from three different cell cultures. The percent of dye coupling in each cell clump was calculated by normalizing the number of cells where LY was detected with the total number of cells in the clump. The numbers on top of the bars correspond to the number of cell clumps injected. Lines correspond to SE. In cell cultures of 8 h, most of the cells were coupled (93 ± 3.09 SE), whereas the fraction of dye-coupled cells decrease importantly in cell cultures of 24 (46 ± 6%) and 36 h (21 ± 2.0%).

Pérez-Armendariz et al., 1995a). Whether luteinizing hormone (LH) and human chorionic gonadotropin hormone (hCG), the main hormones that control steroidogenesis and testosterone release, regulate GJs in Leydig cells has, so far, never been investigated. Here we demonstrate that LH and hCG increase *intercellular communication (ic)* and the expression of Cx43 in mouse cultured Leydig cells. Part of these data have been presented in a preliminary version (Pérez-Armendariz et al., 1995b)

Results

Effect of LH, hCG, and Dibutiryl-cAMP (db-cAMP) on Intracellular Communication (ic)

LH and hCG are the main hormones that control testosterone secretion (Catt et al., 1980; Segaloff and Ascoli, 1993). As an initial step to test if sustained stimulation with secretagogues increases GJs in Leydig cells, the effect of culture time on dye coupling was studied. Lucifer yellow (LY) was microinjected through a patch pipet into one cell of clumps (6–10 cells) mainly formed by Leydig cells (>70%). Figure 1 shows phase-contrast (A and C) and fluorescent micrographs (B and C) of cell clumps cultured in control medium for 8 (A and B) and 36 h (C and D). Leydig cells freshly dissociated were rounded (A), but after being cultured for 36 h, a high percentage of them became elongated (≈80%) (C). Nevertheless, at both culture times, cells were identified by their abundant lipid droplets that were visualized at phase contrast as birefringent bodies (A, C, arrows). In cell clumps cultured for 8 h, LY transferred to most other cells five min after the injection (B). In contrast, in cell clumps cultured for 36 h, after the same period of time, the fluorescent probe did not transfer (D) or if it did it, it was only to a reduced number of its neighbors.

To compare ic between groups of clumps cultured for different times, in each cell aggregate, the number of LY-stained cells was normalized with respect to the total number of cells. Figure 2 illustrates that in cell clumps cultured for 8 h, 93% of the cells were communicated, whereas in cell clumps cultured for 24 h, there was a decrease to 46.5% in the mean percentage of dye-coupled cells (bars amplitude), which further decreased to 21.5% in cell clumps cultured for 36 h.

To test if ic in Leydig cells is upregulated by LH and hCG, LY transfer was compared in control and treated cell cultures of 36 h, where minimum coupling under control conditions was found and possibly larger amplification of the response would be detected. Figure 3 shows phase-contrast (A, B, and C) and fluorescent micrographs (D, E, and F) from a series of cell cultures maintained for 36 h in the presence of 100 ng/mL LH (A and D), 10 ng/mL hCG (B and E), or 1 m*M* db-cAMP (C and F). All treatments completely prevented the decay in dye transfer. Figure 4 shows that when the fraction of dye-coupled area was compared in different cell clumps, db-cAMP, LH, and hCG induced an important increase in the mean fraction of dye-coupled cells of 87, 72, and 98%, respectively, with respect to 21.5% found in controls.

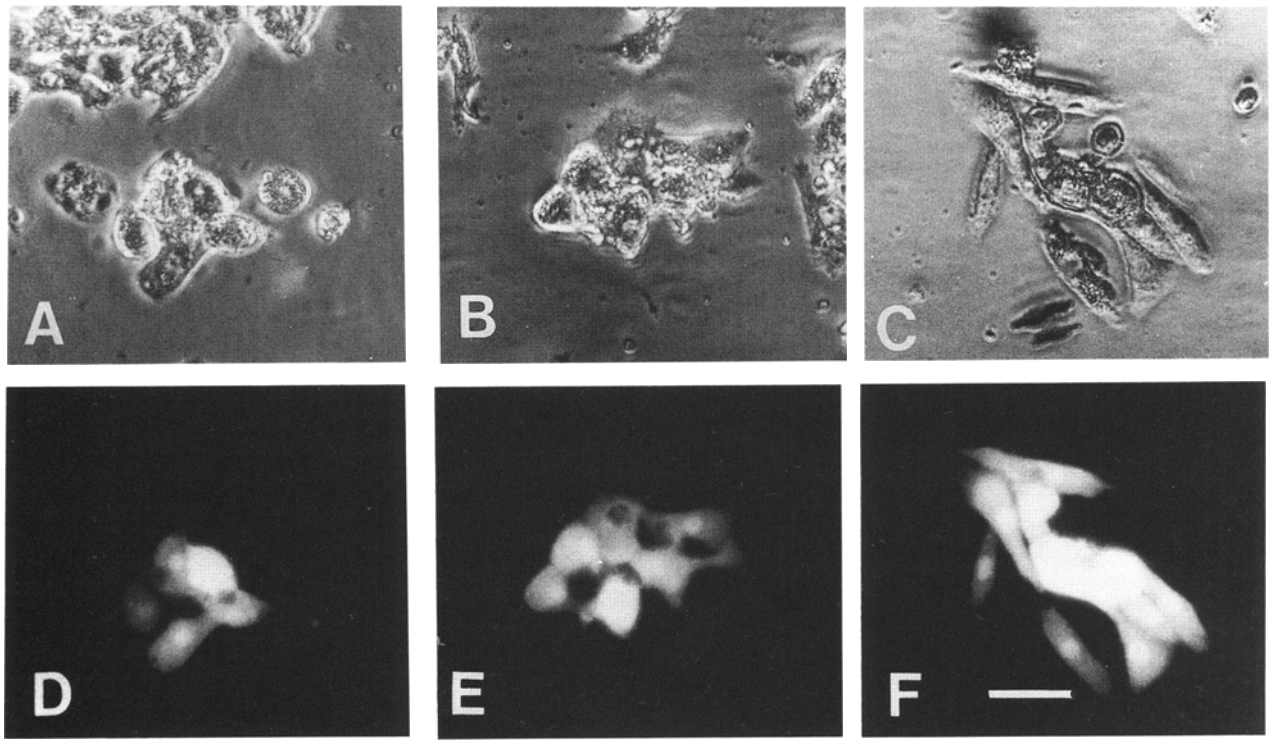

Fig. 3. LH, hCG, and db-cAMP prevent the decay in dye coupling induced by culture time. **(A–C)** Phase-contrast and **(D–F)** fluorescent micrographs of Leydig cell clumps cultured for 36 h. The decay in dye transfer detected in cells cultured for this period of time under control conditions (*see* Fig. 2.) was prevented when cells were maintained in the presence of 100 ng/mL LH (F), 10 ng/mL hCG (D), or 1 m*M* db-cAMP (E). Calibration bar 20 μm.

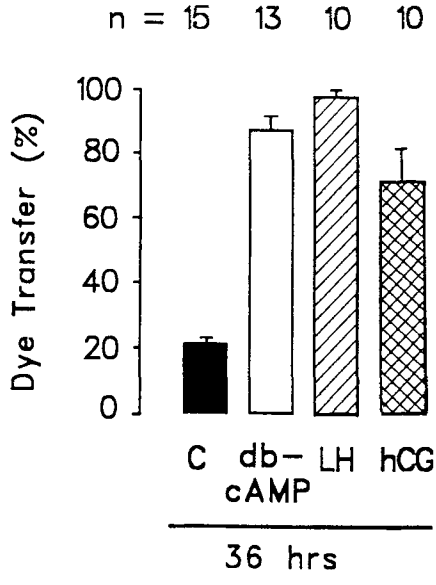

Fig. 4. Percentage of increase in dye coupling induced by LH, hCG, and db-CAMP in cell cultures of 36 h. Illustration of cell clumps from three different cell cultures. Bars, lines, and numbers on top of the bars as in Fig. 2. An important increase in the mean fraction of dye-coupled cells was found in cell cultures maintained in the presence or 1 m*M* dh-cAMP (87 ± 4% SE), 100 ng/mL LH (98 ± 2.0%), or 10 ng/mL hCG (72 ± 10%) compared with cells cultured for the same period of time under control conditions (21.5 ± 2.0%).

Effects of LH, hCG, and db-cAMP on the Expression of Cx43

Cx43 is the only GJ protein identified in Leydig cells to date (*see* Introduction). To test if dye-coupling decay in long-term cultured Leydig cells results from alterations in Cx43 expression, we monitored the effect of culture time on the expression of this GJ protein. Figure 5 shows the phase-contrast (A, C) and immunofluorescence (IF) micrographs (B, D, E, F, G, H) of cell clumps cultured for 8 (A, B, E) and 36 h (C, D, F, G, H) after incubation with anti-Cx43 serum. In cell clumps cultured for 8 h, cell-membrane appositions of most Leydig cells were found to be labeled over a clear intracellular background (B); the amount of labeling was similar to that found *in situ* (Pérez-Armendariz, et al., 1994). In cell cultures of 24 h, Cx43 labeling was reduced (not shown) This reduction was much more evident in control cell cultures of 36 h (C, D) where an important decay in Cx43 labeling was found (≈80%). At this last culture time, in some of the cells, a slightly higher intracellular diffuse fluorescent background was found, and in some others, intracytoplasmic bright spots were detected (D, open arrow). Nevertheless, at some cell-membrane appositions, punctate labeling was still detected (D, closed arrow). Thus, decay in dye coupling was temporally correlated with decay in expression of Cx43.

To determine if hormones also prevented decay in Cx43 expression, IF studies were done in control cell cultures

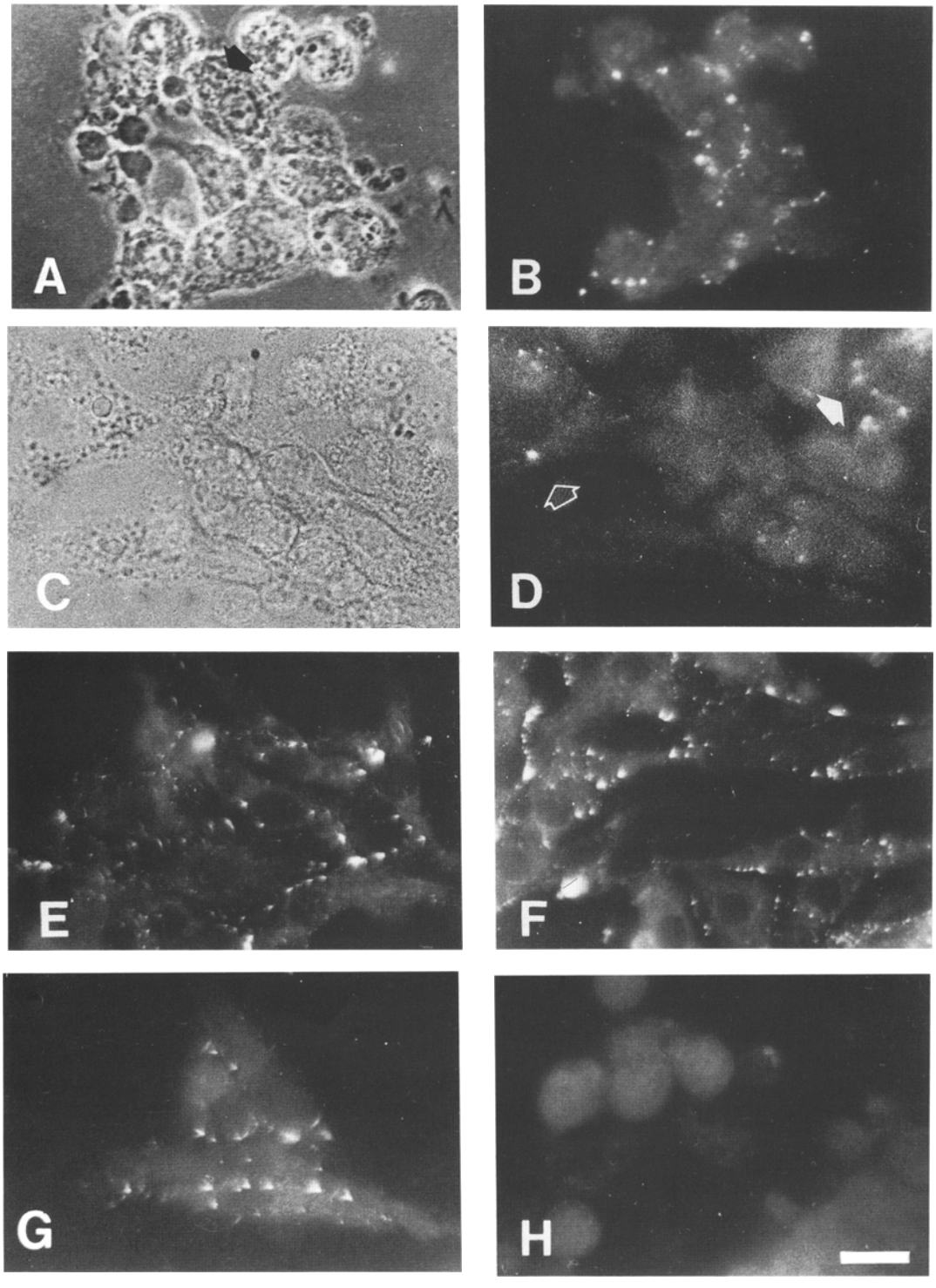

Fig. 5. Decay of Cx43 induced by culture time is prevented by LH, hCG, and db-cAMP. Phase-contrast **(A, C)** and immunofluorescent (IF) micrographs **(B, D–H)** of cell cultures maintained under control (A–D, H) or treated conditions (E–G) for 8 h (A, B, H) or 36 h (C–G), after their incubation with an affinity-purified antiserum against aa 346–360 of Cx43. In cell cultures of 8 h, cell aggregates that exhibit lipid droplets at phase contrast (black arrows) also showed abundant Cx43-IF labeling at cell-membrane appositions over a clear intracellular background (A, B). As a control, panel H shows that no labeling was found after incubation with preimmune serum. (C, D) In cell cultures of 36 h, Cx43-IF importantly decayed in most of the cells. A diffuse intracellular fluorescent background was detected in some of the cells, and in some others, intracellular bright fluorescent spots were detected (D, open arrow). Nevertheless, in some cells, Cx43-IF punctate labeling was still detected at junctional membranes (D, closed arrows). Addition of LH (100 ng/mL) (E), 10 ng/mL hCG (G), or 1 m*M* db-cAMP (F) for 36 h to the medium completely prevented decay in Cx43-IF. Moreover, under this last condition, the number and size of GJ plaques appear larger than those detected in control cell cultures of 8 h (B). Similar observations were obtained in four other experiments.

and in cultures maintained in the presence of 100 ng/mL LH, 10 ng/mL hCG, or 1 m*M* db-cAMP for 8, 24, and 36 h. In cell cultures of 8 h, IF labeling was abundant, and no differences could be detected between control and treated cells (not shown). However, in cell clumps treated for 24 (not shown) and 36 h, LH, hCG, and db-cAMP completely prevented decay of Cx43 labeling at junctional membranes (Fig. 5E, F, and G). Moreover, for all treatments, the number and size of individual plaques appear even larger than the ones detected in membrane appositions of cell aggregates cultured for 8 h (5 B).

Cx43 is known to be a phosphoprotein, which in most cell systems exhibits at least two electrophoretic mobilities; a 40-kDa band, which corresponds to a dephosphorylated form, and a 43 kDa band, which corresponds to a phosphorylated one (Musil et al., 1990; Crow, et al., 1990; Laird et al., 1991). To confirm the specificity of labeling to Cx43 and the possible regulatory effect of LH, hCG, and db-cAMP on this GJ protein, Western blot studies, using the same anti-Cx43 serum as that used for IF studies, were performed. Figure 6 shows immunoblots obtained after electrophoresis of total proteins from homogenates of cell cultures enriched in Leydig cells maintained under control conditions (lane a) or in the presence of 10 ng/mL hCG (lane b), 100 ng/mL LH (lane c), or 1 m*M* db-cAMP (lane d), as well as from heart (lane e) and liver (lane f) tissues. Bands at ≈ 40 and 43 kDa were detected in Leydig cells and in heart, a tissue known to express Cx43 (Beyer et al., 1987), but not in liver tissue that mainly expresses Cx32 and Cx26 (Traub et al.. 1989). Treatment of Leydig cell cultures with hCG, LH, and db-cAMP increased the relative levels of both bands compared to controls.

Cell Identification and Functional State

To determine the fraction of Leydig cells, cell cultures were processed to detect Δ^5-3β-hydroxysteroid dehydrogenase Δ^5-4-isomerase (3β-HSD). The number of positive and negative cells was determined in several micrographs obtained from different fields for each control and treated cell culture (*see* Methods). In cells cultured for 8 h, about 70% of positive cells were found, as previously reported (Kawa et al., 1987; Pérez-Armendariz et al., 1994, 1995b), and no apparent differences were detected between control and treated cells. However, differences were detected between control (Fig. 7A) and treated (Fig. 7B) cells cultured for 36 h. Figure 7C illustrates that a mean percentage increase in three β-HSD-positive (filled bars) cells was induced by LH, hCG, and db-cAMP that varied between 67 to 84%, compared to 56% detected under control conditions. Differences did not result from distinct numbers of cells present in control and treated cells, since the mean number of cells in 14 fields evaluated for each culture condition was similar in all cases. In addition, these treatments also increased the fraction of strongly with respect to the slightly positive cells compared to controls. These results confirm

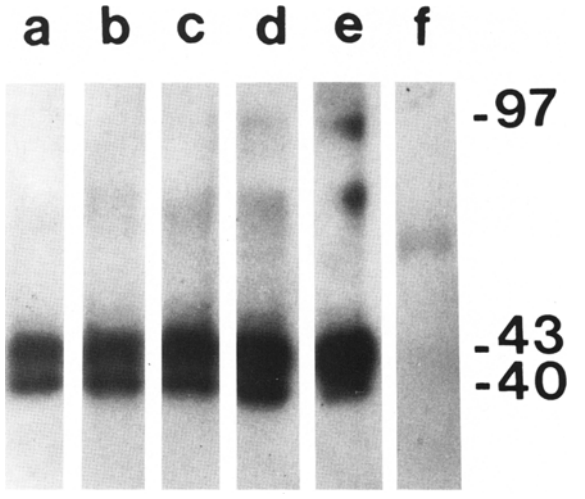

Fig. 6. LH, hCG, and db-cAMP control levels of expression of Cx43 in Leydig cells. Illustration of an immunoblot obtained after electrophoresis of total proteins from homogenates of cell cultures enriched in Leydig cells maintained for 36 h under control conditions (lane a) or in the presence of 10 ng/mL hCG (lane b), 100 ng/mL LH (lane c), or 1 m*M* db-cAMP (lane d), as well as from heart (lane e) and liver (lane f) tissues. Bands of ≈40 and 43 kDa were detected in Leydig cells and in heart (a positive control tissue), but not in liver, which mainly express Cx26 and Cx32. In Leydig cell cultures, relative levels of expression of both bands were enhanced in treated cells compared with untreated ones. Similar results were found in another experiment.

that a large fraction of Leydig cells was found under our culture conditions, and the importance of LH, hCG, and db-cAMP in the control of 3β-HSD activity (*see* Discussion).

In order to know the functional state of cultured Leydig cells, accumulated testosterone levels were measured in the culture medium of cell cultures treated for 8, 24 (8 + 24), and 36 (8 + 24 + 36) h with LH, hCG, or db-cAMP, and in those maintained for the same period of time under control conditions. Figure 7D shows that, after treatment with the indicated secretagogues, the cells significantly increased testosterone secretion, either at 8 (open bars), 24 (not shown), or 36 h (closed bars), compared with basal secretion detected at any time under control conditions. These results indicate that cells are able to increase testosterone secretion in response to steroidogenic hormones along the entire culture period.

Discussion

The main finding in the present work is that we identify for the first time that LH and hCG modulate the expression of GJs and ic between Leydig cells.

Cx43, the Main Connexin Expressed in Leydig Cells, Is Upregulated by LH and hCG

Previously we have proposed that Cx43 is the main connexin mediating ic between Leydig cells, based on an

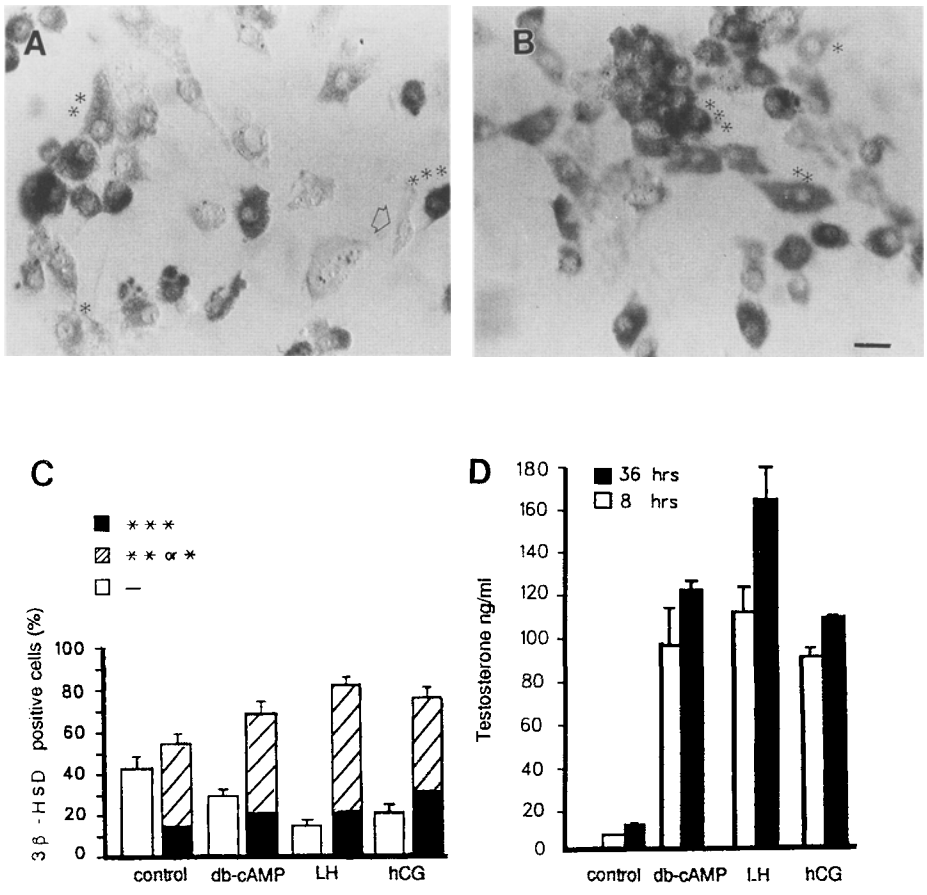

Fig. 7. Identification and functional state of Leydig cells. Phase-contrast micrographs of **(A)** control and **(B)** treated (LH 100 ng/mL) cells cultured for 36 h after staining for 3 β-HSD activity. Round and elongated, strongly (***) or lightly (* or **) 3 β-HSD-positive cells, were identified in addition to some negative ones (arrow). Calibration bar 20 μm. **(C)** Frequency histogram of 3 β-HSD-positive and negative cells detected in cells treated and maintained under control conditions for 36 h. The mean cell number for 14 different fields selected at random was similar in control (53.6 ± 14 SD) and in db-cAMP-(54.8 ± 15.8), LH-(55.0 ± 7.3), or hCG-(42.5 ± 11.3) treated cells Bars amplitude represent the mean percentage of strongly (black bars) and lightly (dashed bars) 3 β-HSD-positive cells or negative (open bars) cells, from a representative experiment. Lines represent SE, LH, hCG, and dh-cAMP induced an increase in the fraction of slightly and strongly 3 β-HSD positive cells that varied between 67 and 84% compared to 56% found in control cell cultures. Similar results were found in another experiment. **(D)** Testosterone secretion in cell cultures of 8 and 36 h. Bar amplitudes represent mean accumulated testosterone levels detected in the culture media from three sister cell dishes for each experimental condition. Lines represent SD. Addition of LH, hCG, or db-cAMP significantly stimulated testosterone release either in cells cultured for 8 (open bars) or 36 (8 + 2:4 + 36) h (black bars). Similar findings were found in another experiment.

immunological and electrophysiological study of GJs in isolated pairs of cells (Pérez-Armendariz et al., 1994). Here we provide further evidence to support this idea, since we found that there is a close correlation between the decay in Cx43 labeling in junctional membranes with the decay in ic in cell cultures of 24 and 36 h. Downregulation of connexin expression has been found under culture conditions (Stutenkemper et al., 1992), and it may result from alteration in GJ gene expression owing to the loss of hormones, extracellular matrix elements, paracrine factors, and other specific contacts between the cells normally present *in situ* (for review, *see* Stagg and Fletcher, 1990, Rosenberg et al., 1993). LH and hCG are trophic hormones whose long term effects involve protein synthesis (for review, *see* Catt et al., 1980; Segaloff and Ascoli, 1993). Thus, it was likely that their absence or low concentration in the control culture media may have altered the expression of Cx43. Indeed, as shown here, the restitution of these hormones in the culture media results in maintenance of direct cell-to-cell communication and Cx43-IF labeling at cell-membrane appositions demonstrating that they play an important role in regulation of GJ expression in this cell system.

Here, it is also concluded that LH and hCG effects on Cx43 expression and ic result from their effects on Leydig cells for three reasons. First, a considerable enrichment in 3β-HSD-positive cells was found in treated cell cultures of 36 h (≈77%). This fraction contrasted with the reduced percentage of 3β-HSD positive cells (56%) detected in cell cultures maintained for the same period of time under control conditions. However, differences were not

accounted for by distinct number of cells present under control and treated conditions, and therefore, they must have resulted from an increase in the number of cells where this enzyme was detected. It is likely that LH, hCG, and db-cAMP have increased the amount of β-HSD produced by each cell, as has been found previously in Leydig cells from other species (O'Shaughnessy and Payne 1982; Kenney and Masson, 1992). Second, ic was evaluated only in selected cell clumps formed mainly by cells that exhibit abundant cytoplasmic lipid droplets at phase contrast, a characteristic of Leydig cells. Third, the expression of LH-hCG receptors in testicular cells other than Leydig cells has, to the best of our knowledge, not been demonstrated. Nonetheless, with the present studies, we cannot rule out that other paracrine factors might also coparticipate in regulation of ic and Cx43 expression, or that LH-hCG effects on ic and Cx43 expression in interstitial cells do not have secondary effects in GJs of other cultured testicular cell types. Future studies will address these aspects.

Possible Mechanisms of LH and hCG Stimulatory Effects on GJ Expression in Leydig Cells

Results presented here indicate that LH and hCG effects on GJs in Leydig cells are being mediated by cAMP, since db-cAMP was found to mimic LH and hCG effects, preventing decay in ic and Cx43 expression with a similar temporal course.

LH and hCG are known to bind to the same receptor and to increase rapidly (min) intracellular levels of cAMP ($[cAMP]_i$) and kinase A (for review, *see* Segaloff and Ascoli, 1993). cAMP is one of the few molecules that have clearly been found to affect GJs in a variety of target cells and types of connexins. In most cell systems, its effects have been found to be stimulatory (for review, *see* Stagg and Fletcher, 1990), but inhibitory effects have also been reported (Lasater, 1985; Cole and Garfield, 1986). cAMP actions have been found to involve changes in the transcription (Metha et al., 1992) and translation rate of connexin genes and/or stabilization of its mRNA (Saéz et al., 1989; Stagg and Fletcher, 1990), as well as changes in the permeability (Flagg-Newton, et al., 1981; Wiener and Lowenstein, 1983) and conductance (Hax et al., 1974; De Mello, 1984; Saéz et al., 1986; Burt and Spray, 1988) of GJ channels. Owing to its multiple sites of actions, its effects have been found to occur within a wide range of time that varied between minutes (gating) up to several hours (long-term modulation) and even through days. The exact mechanisms underlying cAMP large variations in time of action (days) and direction of regulation (stimulatory or inhibitory) between different cell tissues that mainly express a common type of connexin are unknown, but have been proposed to result from cell-tissue-specific regulation of connexins (Stagg and Fletcher, 1990).

Stimulation of LH/hCG receptor has been found to stimulate or inhibit GJs expression in other cell tissues (Bjersing and Cajander, 1974; Browne and Wiley, 1979; Larsen et al., 1981, Cronier et al., 1994). To date, two different GJ gene products, Cx43 (Ambrus and Rao, 1994; and *see* Results) and Cx32 (Valdimarsson et al., 1993), have been found to be regulated by these hormones, although the mechanisms of their regulation are unknown. In Leydig cells, results presented here suggest that cAMP-mediated LH/hCG effects increase the rate of transcription of Cx43 and/or stabilize its mRNA, since in cells treated for 36 h with LH, hCG, and db-cAMP, levels of 40- and 43-kDa bands were found to be greater than the ones detected in controls. In addition, it is also possible that these hormones enhance Cx43 half life in the cell membrane, either by increasing its insertion and/or assembly as GJ plaques or by inhibiting its removal, since first, their absence in the media was associated with detection of intracellular bright spots and decay in IF labeling at cell-membrane appositions, and second, the number and size of IF-Cx43-labeled plaques in cells treated for 24 and 36 h were relatively larger than those detected in controls at 8 h. Future experiments will confirm whether these mechanisms exist in Leydig cells.

Possible Functional Role of LH and hCG Modulation of GJs in Leydig Cells

Prolonged stimulation with secretagogs has been found to increase the synthesis of secretory products as well as the expression of connexins in peptidergic secretory cells (Meda et al., 1979; Int' Veld et al., 1985; Pérez-Armendariz et al., 1995a). Here we have tested this hypothesis in a steroidogenic cell type, and found that long-term stimulation with LH, hCG, and db-cAMP (>24 h) induced an increase in GJs and cell-to-cell communication. In addition, we showed that these treatments, as is already well known, increase testosterone synthesis as indicated by the significant increase in the number of 3β-HSD positive cells, as well as by the enhanced testosterone secretion detected during all the stimulation period. Then results presented here demonstrate that both testosterone and Cx43 synthesis are under the control of LH and hCG. These findings raise the possibility that long-term stimulation of testosterone synthesis may require an increase in ic and GJ expression.

Here we also showed that shorter stimulation periods (8 h) induce a significant enhancement in testosterone production without a detectable increase in GJ or intercellular communication. However, since at this culture time, levels of Cx43 and ic in Leydig cells maintained under control conditions were already close to maximum, these results suggest that possible LH/hCG effects on GJs were not detected with the present methods at this stimulation period, and their study might require the use of other techniques sensitive enough to detect slight changes in the number or gating of GJ channels. Alternatively, these results suggest that longer periods of stimulation with LH-hCG than those required to increase testosterone synthesis may be required to enhance Cx43 expression. Further studies will be

required to clarify this aspect. Nonetheless, since, as shown here, ic remains extensive in the presence of hormones, a possible early role of GJs may be to allow a rapid transfer of changes in $[cAMP]_i$ induced by LH-hCG (Lawrence et al., 1978; Murray and Fletcher, 1984; Fletcher and Greenan, 1985) between a possibly heterogeneous population of Leydig cells (Payne et al., 1980) synchronizing testosterone release from the gland.

Methods

Cell Preparation

Cultures enriched in Leydig cells were obtained using a modification of the technique described by Kawa (1987). Testes were dissected from adult CDI mice, and the tunica albuginea was removed. Three testes were placed in 10 mL of Dulbeco's Modified Eagle's Medium (DMEM) in 50-mL tubes and were gently shaken for 4 min at room temperature until the seminiferous tubules became slightly loose, but not separated. The resulting exudate was enriched in isolated and small aggregates of Leydig cells (≈70–80%, *see* Results). Cells were washed three times by centrifugation. The final pellet from a number of testis was suspended in a small volume of DMEM to adjust a density of 1.2×10^6 cells/mL. Then, 1 mL of cell suspension was plated in glass coverslips and maintained in DMEM with 10% fetal bovine serum for 8, 24, and 36 h in 95% O_2 and 5% CO_2 at 34°C before use.

This isolation technique has some relative advantage over Percoll and Ficoll density centrifugation methods for the purpose of the present study. Because of its briefness and gentleness, most of the yielded cell clumps were mainly natural aggregates of Leydig cells that came apart as groups during the dispersion instead of being formed by reaggregated individual cells. Under these conditions, natural GJ anatomy is better preserved than in reaggregated cells.

Cell Treatment

In each experiment, control and cells treated for 8, 24, and 36 h were processed in duplicate. LH, hCG, or db-cAMP was added to the culture medium when cells were plated. In cell cultures of 24 and 36 h, culture media were renewed at 8 and 24 h.

Cell Identification

After the desired time in culture, cells were washed by transferring the coverslips to a dish containing Krebs Ringer solution where they were gently shaken 5–10 times in order to eliminate detritus and nonattached remaining cells. This step was repeated three times. After the rinsing period, cells were identified by staining for the specific steroid enzyme 3β-HSD, as previously described (Kawa, 1987). To evaluate the number of positive stained cells, for each duplicate of control and treated cell cultures in two different experiments, seven fields were selected at random and photographed under phase-contrast microscopy at 60×. Staining was classified as negative (–), slightly positive (*) or (**). and strongly positive (***) independently by two individuals. For each experimental condition, ≈2000 cells were evaluated. For dye-coupling experiments, Leydig cells were identified by their abundant cholesterol-containing granules that were visualized at phase contrast as birefringent bodies (Christensen, 1975).

Dye Coupling

Cells were washed as indicated above and maintained in Krebs Ringer media with the following composition: 5 m*M* KCl, 155 m*M* NaCl, 1.0 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 2.5 m*M* $NaHCO_3$, 5 m*M* glucose, and 10 m*M* HEPES at pH 7.2., LY (4% in 100 m*M* LiCl, pH 7.16) was injected by diffusion through a patch pipet for 5 min into a cell of clumps (6–10) mainly formed by Leydig cells (>70%). After this time, the number of positive LY-stained cells was evaluated by using conventional fluorescence microscopy with FITC excitation and emission filters, on an Olympus inverted microscope. Injections were done within an hour after the dish was removed from the culture atmosphere, at room temperature.

Antisera

For Cx43 immunostaining, an affinity-purified rabbit antiserum against an oligopeptide corresponding to amino acids (aa) 346–360 in the C-terminal cytoplasmic domain, was used (Yamamoto et al., 1990). Detection of Cx43 antisera was achieved with a goat antirabbit IgG labeled with FITC (Kirkegaard and Perry Lab., Inc.. Gaithersburg, MD).

Immunocytochemical Localization of Anti-Cx43 Serum

Cultured cells were washed three times in normal saline and fixed for 20 min in 70% ethanol at –20°C. Cells were preincubated with 0.1% albumin IgG-free (Sigma Chemical Corp., St. Louis, MO) in phosphate-buffer saline (PBS) for 1 h to reduce nonspecific binding and incubated with primary antibody overnight at 4°C at a final dilution of 1:100. Then, cells were washed with PBS and incubated with FITC-secondary antibodies for 1 h. After several washes, the coverslips were mounted using 0.5% *p*-phenylene diamine in 33% glycerol. In each experiment, control and treated cells were processed in duplicate. Cell labeling under the different culture conditions was compared in at least three different experiments. For each dish, 10 fields selected at random were examined and photographed under phase optics at 60X in transmitted light and epifluorescence. Control tissue sections were processed in a similar manner.

Immunoblotting

Cells cultured for 36 h were harvested by scraping in PBS containing 1 m*M* phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Corp.) to inhibit proteolysis. Cells were centrifuged, and the supernatant was discarded. The pellet was resuspended in 1 m*M* PMSF in PBS and sonicated at 4°C. Total proteins from mouse heart and liver homogenates were prepared in a similar manner. The pro-

tein content of the resuspended pellets was determined by using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) vs BSA standards. Fifty micrograms of total protein from each sample were loaded on each lane of 12.5% sodium dodecyl sulfate (SDS) polyacrylamide gels for electrophoresis. Gels were transferred to nitrocellulose paper (2 h at 300 mA), blocked in 5% nonfat milk in Tris-buffered saline (TBS), pH 7.4, and subsequently incubated with the affinity-purified antibody against Cx43 (2 h, room temperature) at a final dilution of 1:1000 in 5% nonfat milk in TBS. Membranes were washed with PBS (2 h), and the antibody was visualized by conjugation to protein A linked to ^{125}I (ICN Biochemicals, Irvine, CA) and autoradiography.

Controls

We have recently characterized antiserum against aa:346–360 of Cx43 in mouse testicular cell cultures and section (Pérez-Armendariz et al., 1994). Nonetheless, in each IF experiment, positive (heart) and negative (liver) control tissues were incubated with anti-Cx43 serum, in parallel to cell cultures enriched in Leydig cells. As expected, anti-Cx43 serum labeled Leydig and heart, but not liver cell-membrane appositions, and incubations of Leydig cells and control heart tissue with preimmune serum did not resulted in labeling (*see* Results).

Radioimmunoassay

The concentration of testosterone in the incubation media was determined by radioimmunoassay (Castro and Romano, 1994), each in duplicate. The testosterone cross-reacted 18.8% with 5-α-dehydrotestosterone and 1.3% with 5-α-Androstane-3α-17β-diol. The antiserum was supplied by ICN Biochemicals. Cells were maintained in 1 mL of control or treated culture media. At 8 h, the medium from all cell dishes was collected and used for determination of testosterone, and then replaced by the same volume of control or treated fresh medium. This step was repeated at 24 and 36 h.

Chemicals

LH from mouse was obtained as a gift from the National Pituitary Program, N.I.H., Hormonal bank. hCG, db-cAMP, and reactives used for detection of 3β-HSD were purchased from Sigma.

Acknowledgments

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Protein Kinase A and Protein Kinase C Differentially Regulate Steroidogenesis in Human Ovarian Thecal Tumor Cells

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The stimulatory role of protein kinase A in thecal cell steroidogenesis is well documented, whereas the role of protein kinase C is not well defined. In this study, using monolayer cultures of human ovarian tumor cells that are steroidogenically similar to thecal cells, we examined the effects of the protein kinase C activator tetradecanoylphorbol-13-acetate (TPA) on steroidogenesis and the expression of 17 α-hydroxylase cytochrome P450 (P450c17), 3β-hydroxysteroid dehydrogenase (3βHSD), and cholesterol side-chain cleavage cytochrome P450 (P450scc). Cells were uniformly plated and grown to confluence prior to experimental treatment in serum-free medium. Treatments were control, forskolin (10 μ*M*), TPA (0.01–1000 n*M*), and TPA with forskolin. Treatment with TPA alone for 24 h had little effect on basal steroid production, enzyme activities, or mRNA levels. However, when added with forskolin, TPA augmented progesterone production in a concentration-dependent manner. In contrast, TPA inhibited forskolin stimulation of androstenedione production and P450c17 activity. To define better the mechanism of TPA action, Northern analysis of P450c17, P450scc, and 3βHSD mRNA was accomplished using total RNA isolated from cells treated for 24 h. 3βHSD mRNA was increased by forskolin and was not significantly inhibited by treatment with TPA. P450c17 mRNA, however, was suppressed to near undetectable levels by TPA at doses as low as 1 n*M*. In addition, P450scc mRNA expression was inhibited in a manner similar to that seen for P450c17 mRNA. In summary, activation of the protein kinase A pathway increases expression of 3βHSD, P450c17, and P450scc in this thecal cell model. Simultaneous activation of protein kinase A and protein kinase C enhances progesterone production while decreasing androstenedione production and the levels of mRNA encoding P450c17 and P450scc. This differential regulation of steroidogenesis suggests that protein kinase C may play a role in decreased androstenedione production during thecal cell luteinization.

Key Words: 17α-hydroxylase; human theca; ovary; androstenedione.

Introduction

Although there is much information available on the biosynthesis of steroid hormones in thecal cells and their control by gonadotropin (Tsang et al., 1979; Erickson et al., 1985; Hillier et al., 1994), the detailed interaction between hormones and nongonadotropic factors leading to the production of steroids in the thecal cell is not clearly delineated. The role of cyclic adenosine 3', 5'-monophosphate (cAMP) in the stimulation of androstenedione biosynthesis is well recognized. Through activation of protein kinase A, cAMP increases acute steroidogenesis and the capacity of thecal cells to produce additional steroid by increasing steroid-metabolizing enzyme expression (Hedin et al., 1987; McAllister et al., 1989; Demeter-Arlotto et al., 1993; Magoffin and Weitsman, 1993; Rainey et al:, 1996). There is increasing evidence that activation of other second-messenger pathways, including phospholipase C, also has a profound influence on hormone production in the ovary (Knecht et al., 1985; Velduis and Demers, 1986; Leung et al., 1988, 1989; Davis et al., 1989; Wang et al., 1989; Wiltbank et al., 1989; Michael et al., 1993; Steele and Leung, 1993). In this system of signal transduction, an initial step following receptor binding is the breakdown of membrane phosphoinositides into inositol phosphates and 1,2-diacylglycerol. The latter compound, diacylglycerol, is widely recognized to increase the activity of protein kinase C.

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Protein kinase C plays a major role in transmembrane signal transduction (Nishizuka, 1989a,b; Parker et al., 1989). The pleiotropic actions of protein kinase C have been implicated in the regulation of a variety of cellular processes, including proliferation, differentiation, and release of hormones and neurotransmitters (Nishizuka, 1989a,b). The role of protein kinase C in granulosa and luteal cell steroidogenesis has been studied in some detail (Knecht et al., 1985; Velduis and Demers, 1986; Leung et al., 1988, 1989; Davis et al., 1989; Wang et al., 1989; Wiltbank et al., 1989; Michael et al., 1993; Steele and Leung, 1993). In general, activation of protein kinase C inhibits luteal cell production of progesterone and therefore has been suggested to play a role in luteolysis. The role of protein kinase C in ovarian thecal cell steroidogenesis remains less defined, possibly because of the difficulties associated with isolating thecal cells for in vitro studies.

In the present study, we chose to use a human ovarian thecal-like tumor (HOTT) cell model. HOTT cells have retained the ability to secrete androstenedione as well as the expression of 17α-hydroxylase cytochrome P450 (P450c17) (Sawetawan et al.. 1995, 1996; McGee et al., 1995; Carr et al., 1996; Rainily et al., 1996). We hypothesize that the protein kinase A and C pathways can interact to modulate the ability of thecal cells to produce androgens and express steroidogenic enzymes. The purpose of this present study was to determine the role of these two intracellular signaling pathways in regulating thecal cell androgen synthesis in the human ovary. Our data demonstrate that the protein kinase C pathway is a potent inhibitor of the expression of thecal cell P450c17. These data suggest that activation of protein kinase C inhibits thecal cell androgen synthesis and therefore could be involved in luteinization of thecal cells.

Results

Androstenedione Production

Androstenedione production in the culture media was determined by radioimmunoassay after a 48-h incubation. Treatment groups consisted of controls (basal), forskolin (10 μ*M*), and forskolin plus increasing concentrations of TPA (0.01–1000 n*M*). Compared to basal, forskolin increased androstenedione production by two- to threefold (Fig. 1). TPA alone did not alter basal levels of androstenedione secretion, but in the presence of forskolin, TPA caused a concentration-dependent decrease in androstenedione production, reaching maximal suppression at a concentration of (1 n*M*) (Fig. 1). In addition, we conducted a time-course (1–72 h) of the effect of forskolin (10 μ*M*), forskolin (10 μ*M*) plus TPA (100 n*M*), or TPA (100 n*M*) alone on androstenedione accumulation (Fig. 2). Forskolin markedly stimulated HOTT cell production of androstenedione from 6 to 72 h. However, in the presence of forskolin plus TPA, androstenedione accumulation was inhibited. TPA, alone, did not alter basal levels of androstenedione accumulation.

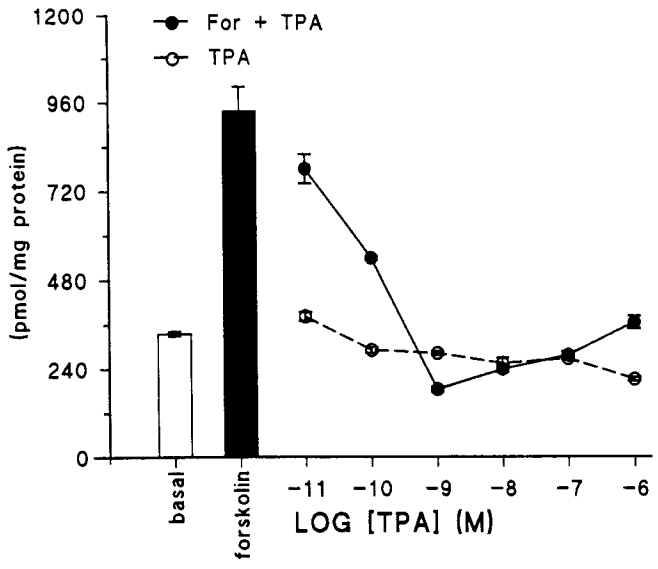

Fig. 1. The effect of various doses of TPA, with and without forskolin on androstenedione production in HOTT cells. Cells were treated for 48 h with and without forskolin (10 μ*M*) or TPA in increasing concentrations (0.01–1000 n*M*). Each data point represents the mean ± SE of replicate dishes (n = 6). Similar results were observed in two additional experiments. —●— For + TPA and —○— TPA.

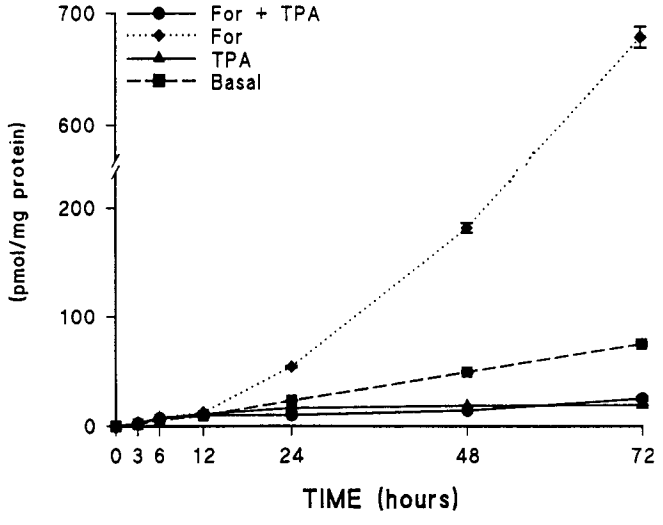

Fig. 2. The effect of forskolin, TPA, and forskolin plus TPA on the production of androstenedione (pmol/mg protein) as a function of time (hours). HOTT cells were incubated in media alone (basal), TPA (100 n*M*), forskolin (10 μ*M*), or TPA plus forskolin for 72 h. Each data point represents the mean ± SE of replicate dishes (n = 6). Similar results were observed in two additional experiments. —●— For + TPA, ····◆···· For, —▲— TPA, and – –■– – Basal.

17α Hydroxylase Activity

We conducted a parallel study after 48 h of incubation on the activity of P450c17 in cells maintained in the presence of forskolin or forskolin plus TPA as described in Fig. 1. In the absence of forskolin, the activity of P450c17 was low and

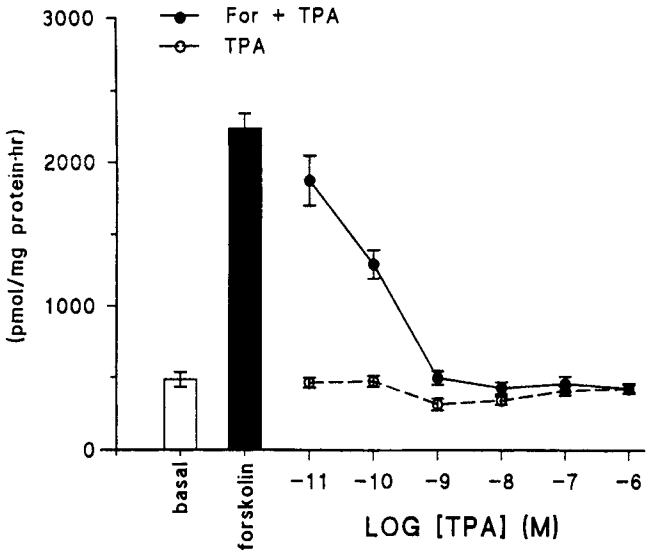

Fig. 3. The effect of TPA with and without forskolin on P450c17 activity in HOTT cells using 2 μ*M* progesterone as substrate. Cells were treated with media alone (basal), TPA (100 n*M*), forskolin (10 μ*M*), or TPA plus forskolin for 48 h. Activity is expressed as pmol/mg protein/h, and each bar or data point represents the mean ± SE of replicate dishes ($n = 4$). Similar results were observed in two additional experiments. —●— For + TPA and —○— TPA.

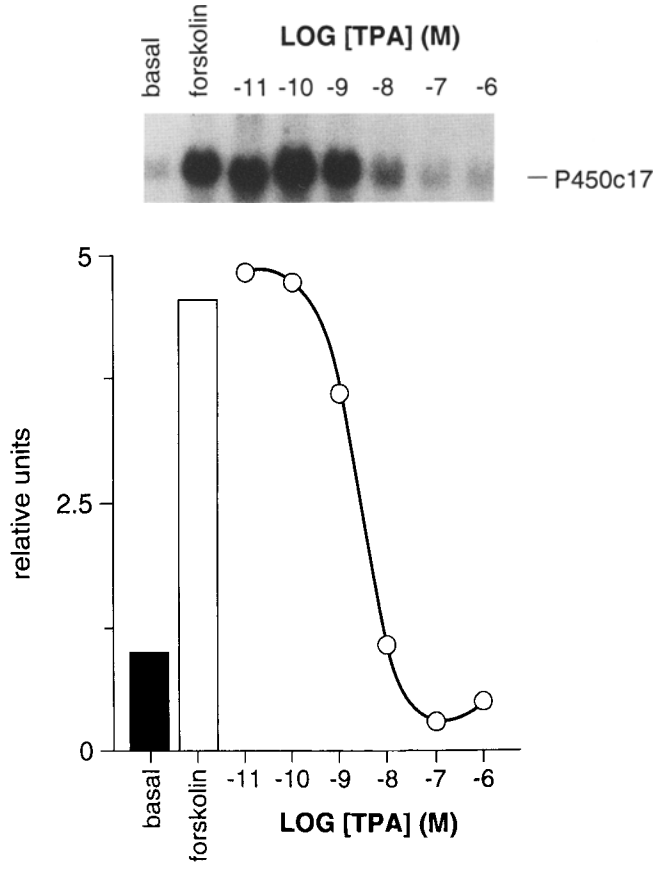

Fig. 4. The effect of forskolin with and without increasing concentrations of TPA on levels of P450c17 mRNA in HOTT cells. Total RNA from HOTT cells probed with cDNA for human P450c17 is presented in the upper panet and the bottom panel represents the levels of mRNA that were quantified as described in Materials and Methods as the amount of radioactivity in each lane normalized to the level of G3PDH mRNA and expressed as fold change relative to untreated cells (basal). These data are representative of two independent experiments.

not affected by TPA. Forskolin, however, stimulated P450c17 activity fourfold compared to basal (Fig. 3). In the presence of TPA plus forskolin, P450c17 activity was inhibited to basal levels in a concentration-dependent manner.

Northern Analysis

Next, we sought to assess the effect of TPA with and without forskolin on the expression of mRNA for P450c17 using the HOTT cell model. Total RNA was isolated from cells treated for 24 h with control (basal), forskolin (10 μ*M*), or TPA in increasing concentrations (0.01–1000 n*M*). Membranes were probed with a cDNA for human P450c17. As presented in Fig. 4, the Northern analysis revealed that forskolin markedly stimulated mRNA for P450c17, whereas TPA in a concentration-dependent manner inhibited forskolin-stimulated P450c17 mRNA expression.

We then assessed the expression of two other key enzymes involved in thecal cell steroidogenesis, P450scc and 3βHSD, using appropriate human cDNA probes as described in Materials and Methods. HOTT cells were incubated in media alone or with LH (100 ng/ mL), forskolin (10 μ*M*), TPA (100 n*M*), or forskolin (10 μ*M*) plus TPA (100 n*M*). The results for P450scc mRNA expression were similar to that for P450c17, namely that TPA inhibited forskolin-stimulated mRNA expression of both enzymes (Fig. 5). In contrast, 3βHSD mRNA expression, which was likewise stimulated by forskolin, was minimally affected by the addition of TPA. Because these cells have lost LH response, no effect of this hormone was observed (Rainey et al., 1996).

Progestin Accumulation and Metabolism

The potent inhibition of P450c17 appeared to alter thecal cell steroid synthesis toward the production of progestins. Therefore, we investigated the effect of forskolin (10 μ*M*) and forskolin (10 μ*M*) plus TPA (100 n*M*) on the metabolism of [^{3}H] pregnenolone. TPA inhibited the forskolin-stimulated metabolism of pregnenolone to 17OH progesterone, 17OH pregnenolone, DHEA, and androstenedione, but markedly enhanced progesterone formation (Fig. 6). Since we observed an increase in [^{3}H] progesterone secretion from the metabolism of [^{3}H] pregnenolone (*see* Fig. 6), we investigated the effect of forskolin (10 μm) with and without increasing concentrations of TPA (0.01–1000 n*M*) on HOTT cell progesterone production (Fig. 7). Again, forskolin-stimulated progesterone accumulation by sixfold compared to basal, however, TPA in a concentration-dependent manner further increased progesterone accumulation up to twofold. However, TPA alone did not affect progesterone accumulation.

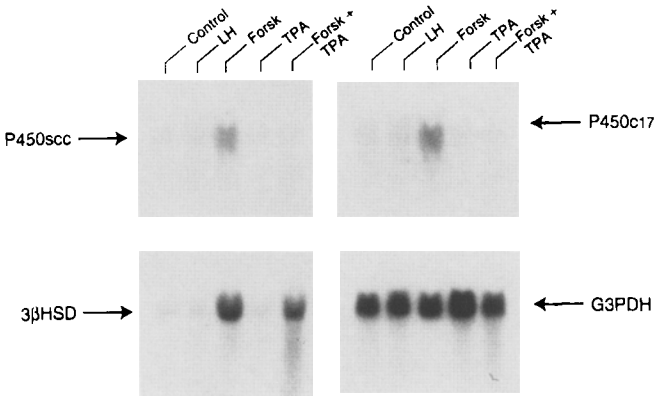

Fig. 5. The effect of LH, forskolin, TPA and forskolin plus TPA on the mRNA expression for P450scc, P450c17, and 3βHSD in HOTT cells by Northern analysis. Cells were treated for 24 h with LH (100 ng/mL), forskolin (10 μ*M*), TPA (100 n*M*), and forskolin plus TPA. Northern analysis was performed as described in Materials and Methods. The expression of G3PDH is included for reference to ensure accuracy of lane loading of RNA. Similar results were observed in two other experiments.

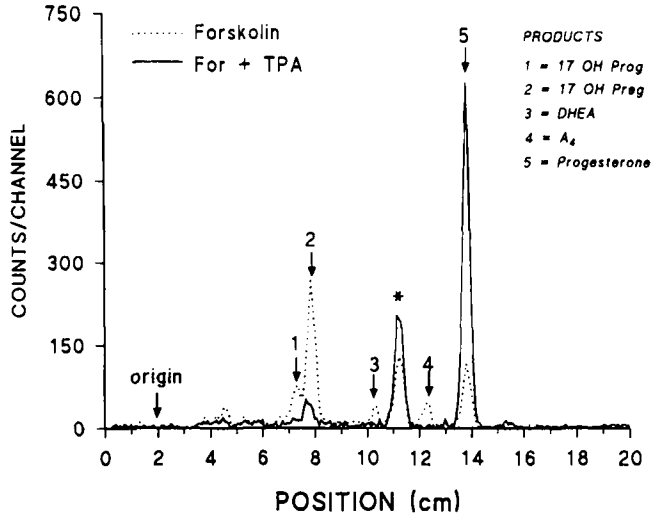

Fig. 6. The effect of forskolin with and without TPA of the metabolism of [^{3}H]pregnenolone in HOTT cells. Cells were treated for 48 h with forskolin (10 μ*M*) or forskolin (10 μ*M*) plus TPA (100 n*M*). At the end of the treatment, pregnenolone (0.5 μmol/L) with [^{3}H]pregnenolone (100,000 dpm/mL) were added to experiment wells for 2 h. Steroids were separated by TLC by comparison to known standards. The asterisk represents the radiolabeled pregnenolone remaining after incubation. These data are representative of two experiments.

Discussion

The level of thecal cell P450c17 expression plays a critical role in regulating the pattern of ovarian steroid production. In the present study, we demonstrate that activation of protein kinase C acts to inhibit HOTT cell androstenedione production and P450c17 mRNA expression. These data suggest a role for protein kinase C in controlling the pattern of steroids produced by the ovary and particularly the theca.

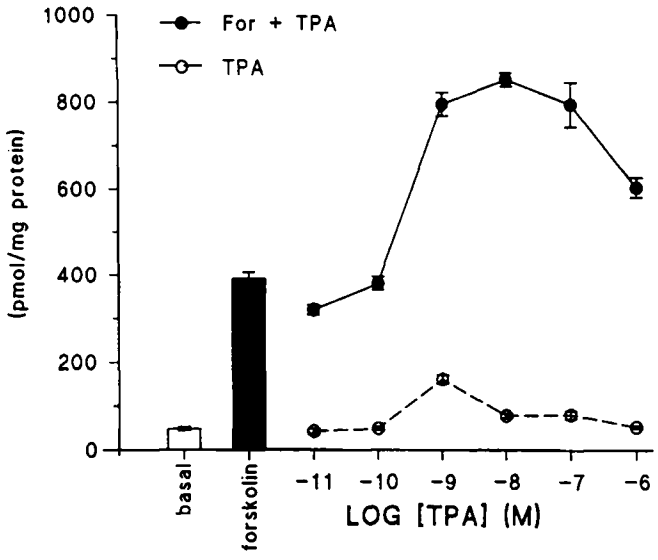

Fig. 7. The effect of forskolin with and without increasing doses of TPA on progesterone production by HOTT cells. Cells were incubated for 48 h in the presence of forskolin (10 μ*M*) with and without varying doses of TPA (0.01–1000 n*M*). The media were analyzed for progesterone content, and presented as mean ± SE of replicates (n = 6) and in pmol/mg protein. Similar results were seen in two additional experiments. —●— For + TPA and —○— TPA.

The maintenance of thecal cell steroid hydroxylase P450s, like maintenance of steroid production, relies on LH. Thecal cells placed in culture rapidly lose expression of P450c17, which can in part be regained by stimulation with LH (McAllister et al., 1989; Demeter-Arlotto et al., 1993). Herein, the HOTT cell model was used to examine the relative role of protein kinase A and protein kinase C in controlling P450c17 and P450scc expression. Since HOTT cells do not have a functional LH receptor, the protein kinase A pathway was activated with forskolin (Rainey et al., 1996). Forskolin increased P450c17 and P450scc mRNA levels in HOTT cells. This confirms our previous findings as well as others who have shown that the protein kinase A pathway is the major activator of steroidogenic P450 expression in thecal cells (Richards, et al., 1986; McAllister et al., 1989; Demeter-Arlotto et al., 1993; Magoffin and Weitsman, 1993; Rainey et al., 1996). To examine the action of protein kinase C in this process, we utilized the protein kinase C activator, TPA. Inclusion of TPA with forskolin blocked the induction of P450c17 and P450scc mRNA. The decrease in P450c17 transcripts was paralleled by loss of P450c17 enzymatic activity and a decrease in androstenedione production. The effects of TPA were clearly not an effect on LH receptors, since forskolin bypasses the necessity of LH receptor activation. The inhibition of P450c17 activity and C19 steroid production by TPA are similar to data recently reported by McAllister and

colleagues using long-term cultures of human thecal cells (McAllister et al., 1994). Rat thecal cells also decrease hormone stimulation of androstenedione production in the presence of TPA (Hofeditz et al., 1988; Simone et al., 1993). It is interesting that TPA treatment of HOTT cells caused an increase in the amount of forskolin-stimulated progesterone production. This shift in steroid profile away from C19 steroids (androstenedione) toward C21 steroids (progesterone) was probably the result of the inhibition of P450c17. The mechanism resulting in an increase of progesterone production in the presence of a TPA inhibition of P450scc transcripts is not clear. However, the activity of P450scc was not examined, but may not be as closely associated to transcript levels of P450c17. These data do suggest that the level of P450c17 expression may be pivotal, at least in this model, in determining the profile of steroids secreted by thecal cells. The physiologic correlate for such a drop in P450c17 expression could be the process of luteinization. In vivo thecal cell expression of P450c17 decreases just after ovulation (Sano et al., 1981; Richards et al., 1986; Hedin et al., 1987; Voss and Fortune, 1993). Although in the human there is a rebound in the expression of P450c17 in the midluteal phase, the mechanism leading to the transient reduction of activity is unknown (Sano et al., 1981; Doody et al., 1990). Thus, one could hypothesize a role of protein kinase C in the process of luteinization.

The protein kinase C pathway has been shown to inhibit P450c17 in adrenocortical cells (Mason et al., 1986, 1995; McAllister and Hornsby, 1987; Rainey et al., 1991; Bird et al., 1992). Although our results demonstrate that TPA decreases the activity and transcript for P450c17, the mechanism for this response is not known. In the adrenal, evidence suggests that protein kinase C acts by reducing transcription of the P450c17 gene (Bakke and Lund, 1992). Hopefully, the HOTT cells will act as a model to define the molecular mechanisms controlling P450c17 gene regulation in thecal cells.

The mechanisms controlling thecal cell protein kinase C activation in vivo are not clear. In the adrenal, angiotensin II appears to be a major regulator of protein kinase C and through that pathway P450c17 expression (Mason et al., 1986, 1995; McAllister and Hornsby, 1987; Rainey et al., 1991; Bird et al., 1992). Studies have demonstrated the presence of protein kinase C within the ovary (Noland and Dimino, 1986; Wetsel et al., 1992). In addition, several nongonadotropic factors thought to influence ovarian steroidogenesis can activate the protein kinase C pathway. These include gonadotropin-releasing hormone, platelet-derived growth factor, prostaglandin $F_2\alpha$, and angiotensin II (Knecht et al., 1985; Velduis and Demers, 1986; Leung et al., 1988; Davis et al., 1989; Wang et al., 1989; Wiltbank et al., 1989; Michael et al., 1993; Steele and Leung, 1993). Further experiments to define the factors that regulate thecal cell protein kinase C will be important in the definition of a physiologic role in ovarian steroidogenesis.

Materials and Methods

Cell Isolation and Culture

A woman presenting with virilization and amenorrhea on examination was found to have a left ovarian mass. Catheterization of the left ovarian vein (with tumor) and right ovarian vein demonstrated excess production of androstenedione and testosterone from the ovary containing the tumor. Dispersed cells (1×10^9 cells total) were isolated from 10 g of the tumor, and aliquots of cells were frozen for future studies. The isolated HOTT cells retained characteristics of human thecal cells in culture, and the isolation, maintenance, and characterization of these cells are described elsewhere (Sawetawan et al., 1995, 1996; McGee et al., 1995; Carr et al., 1996; Rainey et al., 1996). Prior to each experiment, cells were incubated in serum-free medium (DME/F12 containing antibiotics and 0.01% BSA) for 24 h before being rinsed and treated in the same serum-free medium.

Sources of Factors

All chemicals and reagents were obtained from Sigma Chemical Company (St. Louis, MO).

Steroid Measurement

The steroid contents of culture media of cells after various treatments were assayed with DSL radioimmunoassay kits (Diagnostics Systems Laboratories, Inc., Webster, TX). To allow direct comparisons between experiments and account for differential growth between treatment groups, the amount of steroid secreted is expressed as secretion/mg of cellular protein. [^{3}H]Pregnenolone metabolism studies were completed as previously described (Sawetawan et al., 1995; Rainey et al., 1996).

P450c17 Enyme Activity

Enzyme activity for P450c17 was determined by measuring the amount of 17-hydroxyprogesterone produced when cells were presented with 2 μM progesterone as substrate. Activity is expressed as pmoles/mg protein/2 h. The validity of this assay in these cells was determined previously (Rainey et al., 1996).

Protein Determination

Cells were solubilized in Tris-HCl (50 mM, pH 7.4), containing NaCl (150 mM), SDS (1%), EGTA (5 mM), $MgCl_2$ (0.5 mM), $MnCl_2$ (0.5 mM), and phenylmethylsulfonylfluoride (PMSF, 0.2 mM), and stored frozen at −70°C. Protein content of samples was then determined by bicinchoninic acid protein assay, using the BCA assay kit (Pierce, Rockford, IL).

Analysis of mRNA for P450scc, 3βHSD, and P450c17

Cells on 100-mm culture dishes were lysed at 4°C into 1 mL RNAzol B solution (Cinna Biotec, Houston, TX) and transferred to a microfuge tube. Phase separation was achieved by mixing with 0.15 mL $CHCl_3$, incubation at 4°C

for 5 min, and centrifugation (12,000*g* for 20 min at 4°C). The upper phase (0.7 mL) was transferred to a second microfuge tube, and RNA was then precipitated by the addition of 0.8 mL isopropanol and left standing for 1 h at –20°C. RNA was recovered by centrifugation (12,000*g* for 30 min at 4°C), and the recovered pellet was washed once in 75% ethanol (1.0 mL) before drying under air and dissolving in 1 m*M* EDTA, pH 7.0 (0.1 mL). After determination of recovery and purity by measuring absorbance at 260 and 280 nm, samples were precipitated by the addition of 1 mL absolute ethanol and 0.01 mL sodium acetate (3*M*; pH 5.2) and stored at –70°C before analysis.

Samples were separated by electrophoresis on gels containing 1.1% agarose in the presence of formaldehyde. The presence and integrity of the major RNA species were examined under UV light to ensure consistency between lanes. RNA was transferred to a Magna NT membrane (Molecular Separations Inc., Westborough, MA) by pressure blotting (75 psi, 1 h; PossiBlot Pressure Blotter, Stratagene, La Jolla, CA) and crosslinked under UV light. Prehybridization was carried out at 42°C overnight in a final buffer composition of 50% formamide, 5X SSC, 1X PE, and 50 μg/mL transfer RNA (20X SSC contains 3.0*M* NaCl and 0.3*M* trisodium citrate, pH 7.0; 5X PE contains 250 m*M* Tris-HCl [pH 7.5], 0.5% sodium pyrophosphate, 5% sodium dodecyl sulfate [SDS], 1% polyvinylpyrrolidone, 1% Ficoll, 25 m*M* EDTA, and 1% BSA). Hybridizations were performed in the same buffer at 42°C for 16–24 h using antisense probes, which were labeled with [^{32}P] by asymmetric PCR in the presence of [^{32}P]dCTP (Amersham). The blots were then washed in 2X SSC containing 0.1% SDS at room temperature for 15 min, and in 0.1X SSC containing 0.1% SDS at room temperature for 2 x 30 min before drying and exposure to film (Hyperfilm, Amersham).

Probe Preparation

Antisense probes were prepared by PCR in a 50-μL vol under standard conditions, but with the following modifications: forward to reverse primers were added at a 1:100 ratio (0.3 and 30 pmol), the free dCTP concentration reduced 40-fold, and the addition of 50 μCi [^{32}P]dCTP (3000 Ci/mmol, Amersham). Template was added at 10 ng/kb. Labeling was performed through 40 cycles. Incorporation of label was routinely 60–75% by this procedure. Templates and oligonucleotides were as follows: human 17 α-hydroxylase probe template was pCD-17αH (Bradshaw et al., 1987) (provided by Mike Watermann, Vanderbilt University, Nashville, TN), and the forward and reverse oligonucleotides were 5'-GCACCAAGA CTACAGTG-3' and 5'-ACTGACGGTGAGATGAG-3'. 3βHSD probe template was the human Type II cDNA (Lorence et al., 1990), and the forward and reverse oligonucleotides were 5'-CTC TCCAGCATCTTCTG-3' and 5'-TCACTACTTCCAG CAGG-3'. Human P450 scc probe template was a complete cDNA in Bluescript, kindly provided by M. Waterman (Vanderbilt University, Nashville, TN). Forward and reverse oligonucleoltides were 5'-TCTCCTGGTGACAATGG-3' and 5'-CTTGCACC AGTGTCTTG-3', respectively.

Statistical Analysis

Statistical comparisons of means of samples used one-way analysis of variance (ANOVA) with the Newman-Keuls *post hoc* testing. Significance is accepted at the 0.05 level of probability.

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